MAPPING SIMIAN VIRUS 40 MUTANTS BY CONSTRUCTION OF PARTIAL HETEROZYGOTES

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Abstract

Fragments of simian virus 40 DNA, produced by restriction endonucleases *Hin*d (11 fragments) and *Hin*dIII (6 fragments), have been used to map temperature sensitive mutants. One fragment of wild type DNA at a time is mixed with unique linear (*Eco*RI--digested) mutant DNA plus *Hind* or *Hin*dIII--digested mutant DNA. After denaturation and reannealing, some of the product will be multiply nicked circular molecules including a strand of the fragment of wild type DNA. Virions can be produced from the reannealed DNA, but only if the site of mutation is within the fragment of wild type DNA added will a large number of wild type virions arise. In this way, mutants *ts* *A28* and *A30* have been placed in fragment *Hin*dIII-D, *B1* and *B11* in *Hin*dIII-A, and *D101* in *Hin*dIII-E. Further mapping located *ts* *A28*, *A30*, *B1*, *B11*, and *D101* within *Hin*d-I, H, F, G, and E, respectively. The mutants mapped in the same *Hin*d fragments of strain 776 as strain VA45-54.

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Acknowledgment

I thank James Robb and Peter Tegtmeyer for providing SV40 strains, Louise Chow for performing electron microscopy, and Patricia Greene for a gift of *EcoRI* enzyme. Gifts of DNA fragments and enzymes were from Ed Tischer and Kathryn Austin.

Professors Herbert Boyer and Howard Goodman gave invaluable advice and encouragement. David Garfin and JoAnn Leong provided almost daily comment, while Mary Betlach, Richard Meagher, and Patricia Greene at intervals made most helpful suggestions.

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I. Introduction

Restriction enzymes from Haemophilus influenzae serotype d cleave SV40 DNA into distinct fragments which can be separated by electrophoresis (Danna and Nathans, 1971). These fragments have been ordered (Danna, et al., 1973).

I have used such fragments of SV40 DNA, produced by restriction endonucleases Hind (11 fragments) and HindIII (6 fragments) (1), to map SV40 temperature sensitive (ts) mutants. One fragment of wild type DNA at a time is mixed with linear ts DNA plus restriction endonuclease--digested ts DNA; the mixture is denatured and reannealed. Some of the product will carry, annealed to a strand of ts DNA, a strand of the fragment of wild type DNA plus strands of fragments of ts DNA (Figure 1). Virions can be produced from the denatured and reannealed DNA (McCutchan and Pagano, 1968). Only if the site of mutation is within the fragment of wild type DNA added will a large number of wild type virions, detected as plaque formers at high temperature, be produced. In this way five ts mutants have been located in three HindIII fragments and further mapped within five Hind fragments of SV40 DNA.

II. Materials and methods

(a) Virus

SV40 strain VA45-54 and its mutants ts A30, A28, B1, and B11 (2) (Tegtmeyer and Ozer, 1971; Tegtmeyer, 1972), were obtained from Peter Tegtmeyer. Small plaque SV40 (Takemoto, et al., 1966), a derivative of strain 776 (Sweet and Hilleman, 1960; K. Takemoto, personal communication), was from Helene Smith. The mutant ts * 101 (Robb and Martin, 1972) was from James Robb.

(b) SV40 growth and assay

Virus was propagated in CV-1 cells (Jensen, et al., 1964). Temperature sensitive mutants grew at 32°C and wild types at 37°C, all in Eagle's Minimum Essential Medium (Eagle's MEM, Grand Island Biological Co.)

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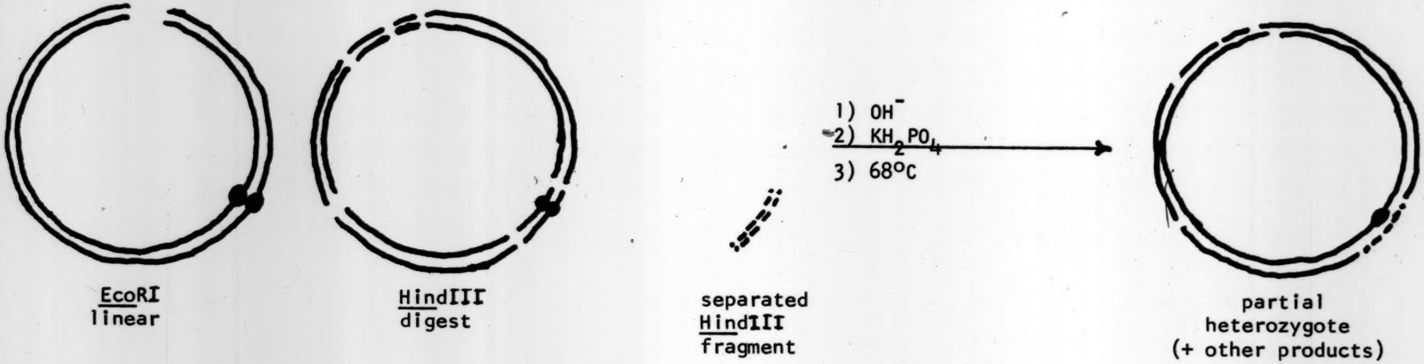
(1) Following the nomenclature of Smith and Nathans (1973), HindII and HindIII are restriction enzymes from Haemophilus influenzae serotype d; the mixture of the two is called Hind (see Danna, et al., 1973, and Smith, 1974).

(2) Nomenclature of Robb, et al. (1972).

Figure 1

Formation of partial heterozygotes by denaturation and reannealing. Solid line: *ts* DNA. Dotted line: wild type DNA. -●-: mutant site.

Figure 1


EcoRI
linear
HindIII
digest
separated
HindIII
fragment
1) OH$^-$
2) KH$_2$PO$_4$
3) 68°C
partial
heterozygote
(+ other products)

supplemented with 4% foetal calf serum, 250 units/ml penicillin, and 250 units/ml streptomycin (all Pacific Biological Co.).

Plaque assays were on TC-7 cells (from James Robb). After two hours virus adsorption, cell monolayers in 5-cm dishes (Nunc) were overlaid with 5 ml Dulbecco's modified Eagle's medium (Smith, et al., 1960; Grand Island Biological Co.) containing 5% foetal calf serum, penicillin and streptomycin as above, 0.35% glucose, and 0.9% agar (Noble agar; Difco). Further 4 ml portions were added after 4, 8, and 10 days incubation at 37 or 40°, after 5, 10, and 20 days at 32°C. The last overlay contained 0.01% neutral red (Grand Island Biological Co.).

(c) SV40 DNA

SV40 DNA was prepared exactly as described by Dugaiczyk, et al. (1974).

(d) Restriction enzymes and digestion of SV40 DNA

Restriction endonuclease EcoRI (Yoshimori, 1971; Greene, et al., 1974), purified through the carboxymethyl cellulose step, was a gift from Patricia Greene.

The HindIII component of Hind restriction endonuclease, purified through the DEAE-cellulose step (Smith, 1974), was a gift from Kathryn Austin. For some experiments it was further purified by diluting, in 0.1 ml portions, to 0.05 M-NaCl with 0.1 ml 0.01 M-potassium phosphate, 0.001 M-EDTA, 0.007 M-β-mercaptoethanol, pH 7.0, and immediately passing over a 0.9 × 4.5 cm column carboxymethyl cellulose (Whatman CM-52) equilibrated with 0.05 M-NaCl in the diluting buffer. Ten min was allowed for equilibration after sample applications (5 to 10 in all), 20 min after the last, and then the column rinsed with 0.05 M-NaCl in buffer. One half ml fractions were collected in tubes containing 0.05 ml 10 mg/ml gelatin and 0.015 ml 5 M-NaCl. The enzyme flows through the column.

The HindII component of Hind endonuclease was a gift from Kathryn Austin or was prepared according to Smith (1974) by gradient elution from phosphocellulose.

Hind enzyme was reconstituted from equal activities of HindII and HindIII. All enzymes were stored at 4°C.

EcoRI reactions were in 100 mM-Tris-HCl, pH 7.4, 50 mM-NaCl, 5 mM-$MgCl_2$, 10 mM-β-mercaptoethanol, and 0.1 mg/ml SV40 DNA, for 20 min at 37°C. HindII, HindIII, and Hind reactions were in 6.7 mM-Tris-HCl, pH 7.4, 60 mM-NaCl, 6.7 mM-$MgCl_2$, 6.7 mM-β-mercaptoethanol, and 0.1 mg/ml SV40 DNA, for 4 hr. at 37°C. Completeness of digestion was checked by electrophoresis into 1.2% agarose gels (Greene, *et al.*, 1974) or 4% acrylamide gels (Danna and Nathans, 1971) using an E-C #470 electrophoresis cell (E-C Apparatus Co.).

For preparative purposes, reactions were made 0.02 M-EDTA, diluted to at least 0.4 ml with H_2O, and treated with an equal volume phenol. After washing the phenol with one half volume TE (0.01 M-Tris-HCl, pH 7.4, 0.001 M-EDTA), the combined aqueous phases were extracted 4 to 6 times with ether, dialyzed against 1/10 x TE, dried under an air stream, and resuspended in TE or H_2O.

(e) *Separated fragments of SV40 DNA produced by* Hind*III* *and* Hin*d*

(i) Hind*III*

Fragments of SV40 DNA produced by *Hind*III digestion were separated by continuous elution gel electrophoresis (Lee and Sinsheimer, 1974). Electrophoresis buffer was 0.04 M-Tris base, 0.018 M-NaCl, 0.02 M-sodium acetate, 0.002 M-EDTA, adjusted to pH 8.05 with glacial acetic acid; the gel was 1.5% agarose (SeaKem) in electrophoresis buffer. Fifty µg dried *Hind*III fragments, containing approximately 5×10^4 cts/min ^{32}P, was resuspended in 0.04 ml 12% glycerol, 0.01% bromphenol blue, applied to the gel, and electrophoresis begun at 120 volts. After 5 min the voltage was reduced to 60 volts and buffer pumped from the bottom collection area at 0.3 ml/min. Two ml fractions were collected and their radioactivity measured by counting Čerenkov radiation. Appropriate fractions were combined and flash evaporated to approximately 1 ml, dialyzed against TE, dried under an air stream, and resuspended in 0.1 ml H_2O. This material was layered over a 5 to 20% sucrose gradient in TE plus 0.1 M-NaCl and centrifuged 5 hr. at 52,000 revs/min in a Spinco SW56 rotor. Fractions were collected and counted, and those with substantial radioactivity combined. Pooled material was dialyzed against 1/10 x TE, dried, and resuspended in H_2O. Fragments were applied to 0.4 x 2 cm columns benzoyl napthoyl DEAE-cellulose (BND cellulose; Serva) equilibrated with 0.3 M-NaCl in TE. The columns were washed with 0.3 M-NaCl in TE, and the DNA then eluted with 1 M-NaCl in TE. After dialysis against 1/10 x TE, drying, and resuspension in 0.2 ml 0.5 M-sodium acetate, 0.6 ml ethanol was added. After overnight storage at -65°C, DNA was pelleted by centrifugation and resuspended in TE.

(ii) Hin*d*

50 or 100 µg *Hind* fragments, containing 1.5 to 5×10^5 cts/min ^{32}P

and in 0.1 ml 25% glycerol, 0.025% bromphenol blue, was electrophoresed into a 0.4 x 15 x 40 cm polyacrylamide gel as described by Danna, et al. (1973). (^{32}P)-DNA bands were located by autoradiography of wet gel, exposing Kodak No-screen film for 1 or 2 days. DNA was eluted by passing excised gel bands through a 3 ml plastic syringe (no needle; for fragments C to K) or by homogenizing with 5 strokes of a Dounce homogenizer (for fragments A and B), then stirring overnight with 3 to 4 volumes 0.3 M-NaCl in TE. Centrifugation at 10,000 x g for 45 min pelleted the gel. Supernatants were applied to BND cellulose columns as before, the columns washed, and DNA eluted with 1.5 M-NaCl, 15% ethanol, in TE. Subsequent dialysis, drying, and ethanol precipitation of DNA were as before.

(f) Denaturation and reannealing

DNA was in approximately 0.001 M-EDTA, 0.03 M-Tris-HCl, pH 7.4. Reaction tubes contained some combination of: 0.09 µg closed circular or linear DNA, 0.45 µg Hind or HindIII--digested DNA, and one or more separated Hind or HindIII fragments in amounts equivalent on a molar basis to 0.45 µg whole SV40 DNA. To ice cold DNA solution in 70 µl was added 10 µl 2.2 M-NaOH, followed after 10 min at room temperature by 20 µl 2 M-KH_2PO_4 (Wetmur and Davidson, 1968). Solutions were then incubated at 68°C for 45 min. After overnight dialysis against 1/10 x TE, DNA was dried under an air stream.

(g) DNA infection

Cells were infected with DNA by the method of McCutchan and Pagano (1968; Pagano, 1971). Dried DNA ((f) above) was resuspended in 0.27 ml TMEM (three parts Eagle's MEM without $NaHCO_3$, one part 0.2 M-Tris-HCl, ph 7.4), then diluted with an equal volume of TMEM plus 1.8 mg/ml DEAE-dextran (Mol. Wt. = 2×10^6; Sigma). After addition of a drop (3 µl) of $CHCl_3$ and 1 hr. at 4°C, the $CHCl_3$ was aspirated and the DNA solution held under a stream of N_2 for 45 sec. This material was used within 2 hr.

Duplicate monolayers of CV-1 cells in 5-cm dishes were washed once with phosphate buffered saline (containing $CaCl_2$ and $MgCl_2$; Dulbecco and Vogt, 1954), inoculated with 0.2 ml DNA--DEAE-dextran mixture per dish, and incubated 20 min at 23°C. After 2 rinses with Hanks' balanced salt solution (also with $CaCl_2$ and $MgCl_2$; Hanks and Wallace, 1949), monolayers were covered with 5 ml Eagle's MEM plus 4% foetal calf serum, penicillin and streptomycin as before, and incubated at 32°C. After 5 days, virions were harvested by twice freezing and thawing, and debris removed by centrifugation at 1,500 x *g* for 15 min.

III. Results

(a) Mapping ts mutants in HindIII fragments

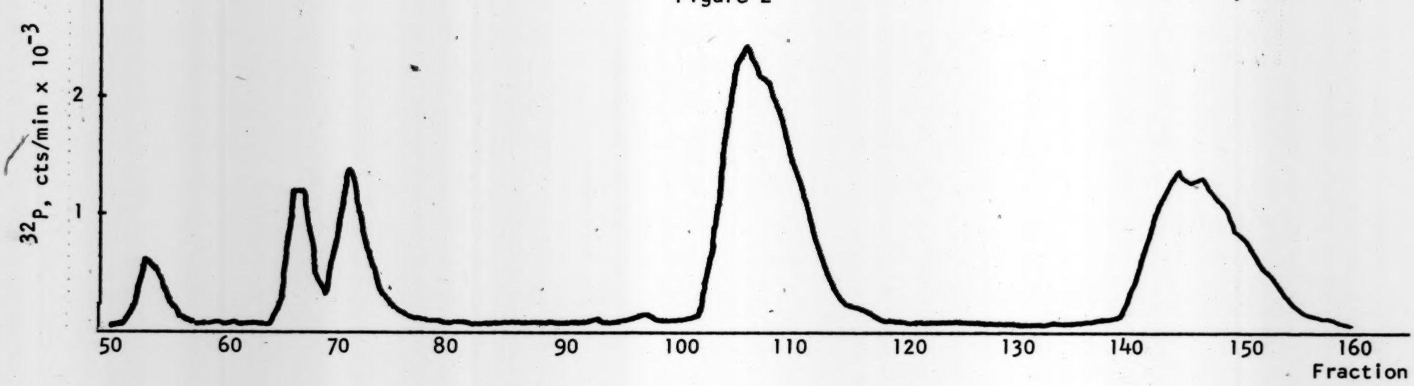
Continuous elution gel electrophoresis of *Hind*III--digested strain VA45-54 SV40 DNA yielded five peaks of radioactivity (Figure 2). The DNA in these was *Hind*III fragments F, E, D, C + B, and A (in order of elution), as shown, after work-up (*Materials and methods*), by electrophoresis parallel to unfractionated *Hind*III digest in the E-C apparatus.

Portions of separated fragments were mixed with *Hind*III--digested and with *Eco*RI--digested (linear) *ts* mutant DNA, with both *Hind*III digest and separated fragment in 5-fold molar excess over linear DNA. Control mixtures were wild type closed circular (form I) DNA, *ts* DNA only, six separated fragments alone, and six separated fragments plus linear *ts* DNA with or without *Hind*III digest. After denaturation and reannealing, the DNA was used to infect CV-1 cells. Virions, which presumably arose through the intervention of cellular repair enzymes, were harvested after one cycle of growth at 32°C. Wild type virions among these were assayed on TC-7 cell monolayers, incubating assay dishes 22 hr. at 32°C and then 10 days at 40°C. This period at 32°C and the one cycle of growth at 32°C were intended to avoid interference by possibly *trans*-dominant *ts* mutants. Table I shows the titers obtained. For each mutant, a particular fragment of wild type DNA gave a high titer of wild type virus. I therefore place mutants *ts A28* and *A30* in *Hind*III-D, *ts B1* and *B11* in *Hind*III-A, and *ts * 101* in *Hind*III-E.

(b) Ts mutants in Hind fragments

Strain VA45-54 DNA was treated with *Hind* endonuclease, and the digested DNA electrophoresed into polyacrylamide gels. Autoradiography revealed 11 bands in the published pattern (Danna and Nathans, 1971; Lai and Nathans, 1974a), so long as the *Hind* had been made up with carboxymethyl cellulose--treated *Hind*III. DNA in gel bands was worked up as described in *Materials and methods* and used in mapping experiments exactly analogous to those done with *Hind*III fragments. Results are in Table II. Again, each mutant can be placed in a particular fragment: *ts B1* in *Hind*-F, *B11* in G, *A28* in I, *A30* in H, and *ts * 101* in E.

Figure 2



Continuous elution gel electrophoresis of HindIII--fragmented SV40 DNA

Table I

Recovery of wild type virus from ts DNA and separated HindIII fragments of wild type DNA. Pairs of numbers give results of two separate experiments.

ND = not done
(a) zero plaques per 0.2 ml is $\leq$ 20/ml with 95% confidence.
(b) wild type DNA was strain 776; fragments a gift from E. Tischer.
(c) fragments not adsorbed and eluted from BND-cellulose.
(d) fragments of wild type DNA had been stored 4 months at 4°C.
(e) HindIII fragments A through F.

Table I

Wild type SV40 from HindIII fragments of SV40 DNA

DNA mixtures			PFU/ml at 40°C				
ts DNA		wild type DNA	ts mutant				
RI linear	HindIII		A28	A30	B1	B11	* 101
+	+	-	(a)≤20; ≤20	(b)220; 20(c)	(b)≤20; ≤20(c)	≤20; ≤20(d)	≤20; ≤20(d)
+	-	A to F(e)	11,000; 6,000	1,100; 1,500	2,150; 2,550	13,500; 750	550; 80
+	+	A to F	6,500; 3,350	1,750; 800	550; 2,300	2,850; 275	1,750; 225
+	+	A	≤20; 10	20; 50	900; 2,550	3,050; 1,250	≤20; ≤20
+	+	B + C	25; 205	ND; ≤20	≤20; 25	≤20; 10	≤20; ND
+	+	D	9,000; 13,500	2,450; 3,450	ND; ≤20	≤20; ≤20	150; 10
+	+	E	10; ≤20	ND; ≤20	≤20; ≤20	≤20; ≤20	1,800; 110
+	+	F	≤20; 5	ND; ≤20	60; ≤20	≤20; ≤20	≤20; 20
-	-	A to F	(≤20)	(145; ≤20)			
-	-	form I	(6.5×10^6)	(2.0×10^6)	(2.0×10^5)	(5.0×10^5)	

Table II

Recovery of wild type virus from _ts_ DNA and separated _Hind_ fragments of strain VA45-54 wild type DNA. Pairs of numbers are results of two separate experiments.

(a) _Hind_ digestion of _ts_ and wild type DNA used _Hind_III not passed over carboxymethyl cellulose (see _Materials and methods_).
(b) In one preparation of separated fragments, the radioactivity expected in band _Hind_-F was in two bands between E and G. The upper, minor, band was called F', the lower F, and the two treated as separate fragments.
(c) _Hind_ fragments A through K.

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Table II

Wild type SV40 from Hind fragments of strain VA45-54 DNA

DNA mixtures			PFU/ml at 40°C				
ts DNA		wild type DNA	ts mutant				
RI linear	Hind		A28	A30	B1	B11	* 101
+	+	-	(a) ≤20; ≤20	(a) 45; ≤20	(a) 5; ≤20	(a) ≤20; ≤20	(a) ≤20; ≤20
+	-	A to K(c)	12,000; 3,100	700; 17,000	9,500; 8,500	15,500; 2,100	600; 1,150
+	+	A to K	8,500; 5,600	3,000; 8,500	8,000; 8,000	8,500; 1,300	700; 650
+	+	A	450; 750	≤20; 45	55; ≤20	85; ≤20	250; 105
+	+	B	5; ≤20	85; 20	5; ≤20	10; ≤20	250; ≤20
+	+	C	≤20; ≤20	5; 25	≤20; ≤20	5; ≤20	≤20; ≤20
+	+	D	≤ 20; ≤20	300 ; 85	≤20; ≤20	≤20; ≤20	50; ~~≤20~~
+	+	E	≤20; ≤20	70; 10	45; ≤20	100; ≤20	1,000; 1,600
+	+	F'(b)	≤20	55	1,850	205	450
+	+	F	10; ≤20	10; 650	750; 9,500	195; ≤20	10; ≤20
+	+	G	≤20; ≤20	≤20; 15	175; ≤20	11,000; 2,150	5; ≤20
+	+	H	500; 30	600; 5,000	≤20; ≤20	≤20; ≤20	≤20; ≤20
+	+	I	10,000; 12,000	55; 15	≤20; ≤20	≤ 20; ≤20	≤20; ≤20
+	+	J	40; ≤20	≤20; 215	≤20; ≤20	≤20; ≤20	≤20; ≤20
+	+	K	30; 260	≤20; 20	≤20; ≤20	≤20; ≤20	≤20; ≤20
-	-	A to K	(≤20; ≤20)				
-	-	form I	(2.0×10^6; 2.3×10^5)		(1.4×10^6; 6.5×10^5)		

(c) *Similarity of SV40 strains 776 and VA45-54*

The order of *Hind* fragments has been determined for SV40 strain 776 (Danna, *et al*., 1973). Two methods were used to compare the sequences of strain 776 with those of strain VA45-54. (1) Heteroduplexes formed with strain VA45-54, strain 776, and with an equimolar mixture of each, were examined by electron microscopy (done by Louise Chow). She found no deletion, addition, or substitution in one strain relative to the other. (2) There remained possibilities such as strain 776 *Hind*-I plus 25 base pairs = strain VA45-54 *Hind*-H and strain 776 *Hind*-H minus 25 base pairs = strain VA45-54 *Hind*-I, *i*. *e*., essentially the same sequences interchanged in gel position by way of deletion and addition too small to be seen by electron microscopy. However, each *ts* mutant mapped in the same fragment of strain 776 DNA (Table III) as strain VA45-54 (Table II). I conclude that at least fragments E, F, G, H, and I are in the same order in the two strains, and therefore that the mapping results presented here may be correlated with results of others (see *Discussion*) based on the strain 776 sequence.

III. *Discussion*

(a) *Possibility of artifact*

Figure 3 shows the order and alignment of *Hind*III and *Hind* fragments, according to Danna, *et al*. (1973). Comparing the figure with Tables I and II, in every case the *Hind* fragment which corrects a mutant is part of the *Hind*III fragment which corrects the same mutant. Also from Tables I and II, *ts* DNA alone does not give wild type virus. Not all mutants map in the same fragment. Finally, the results presented here for mutants *ts* *A28*, *A30*, and *B11* agree with those of Lai and Nathans (1974a) for the same mutants. These considerations argue against artifact, and are consistent with a particular DNA sequence being required to correct a particular mutation.

(b) *Physiological correlates*

ts *A28* and *A30* are "early" SV40 mutants, which affect viral DNA synthesis (Tegtmeyer, 1972); *ts* *B1* and *B11* are "late", *B1* producing empty capsids at high temperature and *B11* giving no virions at high temperature and heat sensitive virions when grown at low temperature (Tegtmeyer and Ozer, 1971; Tegtmeyer, 1972). *ts* * *101*, a *ts* *D* mutant (Chou and Martin, 1974), may be in a late gene which functions early (defective uncoating? See Robb and Martin, 1972, and Chou and Martin, 1975; but note also Robb,

Table III

Recovery of wild type virus from ts DNA and separated Hind fragments of strain 776 wild type DNA.

(a) Hind fragments A through K.

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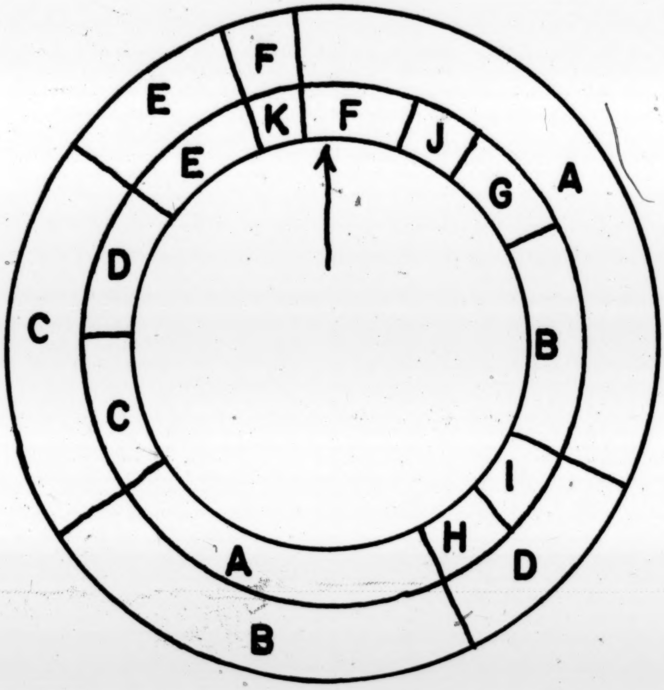
Table III

Wild type SV40 from *Hind* fragments of strain 776 DNA

DNA mixtures			PFU/ml at 40°C				
ts DNA		wild type DNA	ts mutant				
RI linear	Hind		A28	A30	B1	B11	* 101
+	+	-	≤20	≤20	≤20	≤20	≤20
+	-	A to K[a]	1,700	1,400	750	3,000	4,200
+	+	A to K	2,300	1,300	1,450	3,200	10,000
+	+	A	165	≤20	≤20	80	35
+	+	B	≤20	≤20	5	≤20	85
+	+	C	90	≤20	≤20	≤20	80
+	+	D	≤20	≤20	≤20	≤20	45
+	+	E	≤20	≤20	15	25	3,600
+	+	F	≤20	≤20	2,100	≤20	450
+	+	G	≤20	≤20	≤20	3,750	≤20
+	+	H	35	3,700	5	≤20	≤20
+	+	I	9,500	≤20	5	≤20	≤20
+	+	J	≤20	≤20	≤20	≤20	≤20
+	+	K	≤20	50	≤20	≤20	≤20
-	-	A to K	(≤20)				
-	-	form I	(2.5×10^5)				

Figure 3



Hind and HindIII cleavage maps of SV40 DNA. Segments of the inner circle are Hind fragments. Segments of the outer circle are HindIII fragments. The arrow shows the site of cleavage by EcoRI. From Danna, et al.(1973).

1973).

By annealing RNA from infected cells to separated strands of restriction enzyme fragments of radioactive SV40 DNA, "early" and "late" stable SV40 RNA sequences have been mapped (Khoury, _et al_., 1973; Sambrook, _et al_., 1973; Khoury, _et al_., 1975). Also, hybrids of SV40 with adenovirus 2 have been characterized with respect to segment of SV40 DNA carried (Morrow, _et al_., 1973; Lebowitz, _et al_., 1974) and early SV40 antigens produced (Kelly and Lewis, 1973; Lewis and Rowe, 1973).

As might be expected, the 'late' mutants _ts B1_ and _ts B11_, in _Hind_-F and G, are in the "late" RNA region. The "early" mutants _ts A28_ and _ts A30_, mapping in _Hind_-I and H, are in the region associated with "early" RNA and with early SV40 antigens. _Ts * 101_, in _Hind_-E, maps in the "late" RNA region; whether the mutation marks a "late" gene is at present uncertain.

(c) _Genetics of SV40_

The results presented here form part of a genetics involving direct manipulation of DNA (cf. Mertz and Berg, 1974, Lai and Nathans, 1974a and 1974b), classical genetic mapping being difficult because recombination is infrequent (Dubbs, _et al_., 1974). Similar work has been done with bacteriophage φX174 (Hutchison and Edgell, 1971). In these cases one constructs a genotype and then examines the phenotype, the reverse of the classical method. The resulting genetic map then allows comparison of the physiological effects of the mutants with patterns of transcription and of antigen synthesis, all by reference to the restriction enzyme fragment map.

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